MICE PATHOGENICITY OF *PASTEURELLA MULTOCIDA* STRAINS ISOLATED FROM DISEASED AND HEALTHY RABBITS

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ABSTRACT

Pasteurella multocida strains (n=12) were isolated from rabbit carcasses from pathological lesions (n=3) and from the nasal mucosa of clinically healthy (n=5) or diseased (n=4) animals. Mice were challenged intraperitoneally with 5 series of tenfold dilutions and 50% lethal dose was estimated. One strain was highly pathogenic with LD_{50}=1000 CFU. Another seven strains showed LD_{50} between 1.9×10^5 CFU and 2.9×10^6 CFU and were considered as pathogenic or slightly pathogenic. Four strains had LD50 6×10^8 or more CFU and were not pathogenic to mice at all. A set of parameters, including health status and clinical or pathological lesions of sampled rabbit, colony and capsular types of the bacteria, occurrence of neurological signs or systemic infection in mice were evaluated statistically to find any association with the virulence. The circumstances of the isolation were found to be important, as all isolates taken from dead rabbit clustered together, and three of them were also pathogenic to mice. The remaining one was classified being non pathogenic on mice, although a relatively high number of mice were killed with the concentrated inoculum containing 10^7 CFU (but none with the diluted ones). Differences in capsular serotype also showed some interaction with the circumstances. The only two capsular type F strains were collected from healthy rabbits and were separated below 60% similarity into one cluster each. These were also non pathogenic to mice and did not produced neurological signs. A further cluster included 4 strains taken from ill or apparently healthy rabbits, and all had *capA* genotype. The lethality of these isolates to mice was heterogenous, one non-pathogenic, two moderately pathogenic and one pathogenic were found. Considering these results it can be concluded, that ill and clinically healthy rabbits too could spread pathogenic or moderately pathogenic strains, and the proportion of the non-pathogenic strains is not different in the apparently healthy rabbits. Strains isolated from the dead rabbits are characterized by higher mouse virulence level.

Key words: *Pasteurella multocida*, Sanitary status, Rabbit, Mouse, Pathogenecity.

INTRODUCTION

Pathogenicity of *P. multocida* strains isolated from rabbit varies on a very wide range. Knowledge of virulence is important for selection of virulent strains in order to be able to analyze experimentally the efficacy of therapy or vaccination. Yet only few strains have been studied in experimental infections mainly because of the heterogen response of the rabbits infected with the same strain (Rideaud *et al*., 1999). Mouse however is known to be very sensitive to *P. multocida* infection, hence mice intraperitoneal inoculation is the choice not only for isolation from mixed bacterial populations (Laviere *et al*., 1993) but it could be used for the estimation and comparison of strain's pathogenicity too (Boyce and Adler, 2000). Although strains virulent in rabbits were found to be also virulent in mice (Savage and Seldon, 1971; Okerman *et al.*, 1979) only few further results have been published (Collins *et al*., 1983).

The aim of the study was the estimation of the pathogenicity and the comparison of previously characterized *P. multocida* strains which had been collected in the propagation and fattening units in Hungary (at a distance of 32 km) of the same commercial breeding system. Investigation of the

association between *P. multocida* types, mice pathogenicity and the source rabbit's sanitary status was performed.

MATERIALS AND METHODS

Bacterial strains

P. multocida was isolated from nasal mucosa of live rabbits being asymptomatic (n=5) or displaying pasteurellosis signs (n=3) and from lesions found at necropsy (n=4) as shown in Table 1. Capsule production and serogroup, as well as biovar presented here was previously determined and published elsewhere (Virág *et al*., 2008).

Table 1: Source and phenotype of 12 *P. multocida* strains used for experimental infection

	Source rabbits condition and sampled site			Strain's characteristics		
Strain	Exploitation	Lesion	Sampling site	Capsule production	Capsular type	Biotype
1	Broiler	Pneumonia	Lung	+	A	6
3	Breeder	Subcutaneous abscess	Nasal mucosa	+	D	Nt
5	Breeder	Subcutaneous abscess	Nasal	-	A	Nt
9	Broiler	Nothing	Nasal	+	A	1
13	Breeder	Rhinitis	Nasal	+	A	1
18	Broiler	Nothing	Nasal	+	A	1
22	Breeder	Nothing	Nasal	+	A	3
23	Breeder	Nothing	Nasal	+	F	6
26	Breeder	Nothing	Nasal	-	F	6
29	Breeder	Pneumonia	Lung	+	A	1
31	Breeder	Empyema	Chest	+	A	6
32	Breeder	Torticollis	Nasal mucosa	+	A	Nt

Nt=non typable

Assessment of virulence

P. multocida strains were grown in BHI for 24 hours, the achieved concentrations were approximately 10^3 to 10^7 CFU/ml. Exact bacterial numbers checked by direct plate viable counts are presented in Table 2. Broth cultures were diluted in BHI to obtain 4 series of tenfold dilutions.

SPF NMRI mice (13-16 g) were challenged (n=3 per dilutions, N=15 per strain) intraperitoneally with aliquots of 0.5 ml of the appropriate dilutions. Groups of three mice infected with the same dilution of the same strain were kept together. During the 7 days following the infection disease signs and mortality were observed. Mice still alive on day 8th post infection were humaneously killed and *P. multocida* isolation from the hearth blood was attempted. Lung and brain tissues were taken for hystopathological investigation from animals which have been ill during the observation.

Statistical Analysis

All statistical procedures were performed by GenStat software (*VSN International Ltd.*). Median infective and lethal dose (ID_{50} and LD_{50}, respectively) causing morbidity or mortality of half of the mice was estimated by probit analysis. Strains were classified into three pathogenicity groups arbitrarily defined (non-pathogenic, slightly-pathogenic, pathogenic) on the base of the ID_{50} values and hierarchical grouping based on strain characters and pathogenecity parameters were used to arrange infective strains into homogeneous groups. Similarity matrix was calculated with DICE and a dendrogram was constructed by UPGMA procedure.

RESULTS AND DISCUSSION

The pathogenecity of a given strain was initially inferred from the number of disease and death occurences in groups of 3 mice infected with sequentially diluted inoculums (Figure 1). Strains 1, 13 and 31 caused clinical symptoms/death at lower concentrations ($\geq 10^2$ CFU), strains 3, 5, 9, 22 and 32 were infective only at higher ($>10^3$ CFU) doses. Similar result was published by Collins *et al.* (1983) who found that slow growing *P. multocida* isolated from rabbit rhinitis caused mortality only at high infective doses. Remaining 18, 23, 26 and 29 strains showed only very few effects even at the highest concentrations.

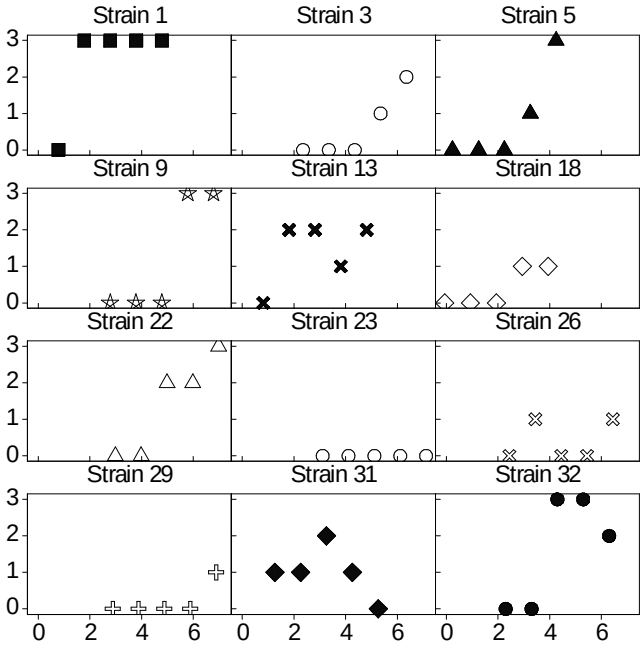

Figure 1: Trellis plot of morbidity and mortality losses caused by experimental infection in mice with 12 *P. multocida* strains. Y axis=number of dead or morbid cases between 3 mice challenged by the same inoculum dose, X axis= Log10 of inoculum concentration

Clinical sings of the disease began with piloerection, dullness, and huddling. Acute phase lethality (Table 2, N=15 mice/strain) changed between 0% and 47%, the highest values were found in the groups infected with strains 1, 9, 13, 22 and 32 (46.7%, 26.7%, 20%, 33.3% and 33.3%, respectively).

Table 2: Inoculum size (concentrated) and LD_{50} estimation of the 12 *P. multocida* strains used for experimental infection. Results of the classification into 3 pathogenecity groups.

Strain	Infection dose[1]	Lethality (%±SE)[4]	Bacteriemia[2]	Neurological signs[3]	Estimated CFU		Classification of virulence
					ID_{50}	LD_{50}	
1	6.0×10^4	46.7 ± 0.13^b	+	0	6.11	1.1×10^3	Pathogenic
3	2.3×10^6	6.7 ± 0.06^{ab}	+	1	2.1×10^6	1.1×10^9	Apathogenic
5	1.8×10^4	13.3 ± 0.09^{ab}	–	0	5.4×10^3	7.7×10^5	Pathogenic
9	6.1×10^6	26.7 ± 0.11^b	+	2	2.4×10^5	6.9×10^6	Slightly pathogenic
13	6.4×10^4	20 ± 0.10^b	+	4	1.1×10^3	1.9×10^5	Pathogenic
18	8.7×10^3	6.7 ± 0.06^{ab}	–	1	2.9×10^4	5.7×10^6	Slightly pathogenic
22	9.7×10^6	33.3 ± 0.12^b	+	1	1.6×10^5	2.4×10^6	Slightly pathogenic
23	1.3×10^7	0^a	–	0	1.1×10^{13}	1.6×10^{17}	Apathogenic
26	2.8×10^6	6.7 ± 0.06^{ab}	+	0	5.3×10^6	6.0×10^8	Apathogenic
29	7.7×10^6	6.7 ± 0.06^{ab}	–	0	1.9×10^8	5.0×10^9	Apathogenic
31	1.8×10^5	20 ± 0.10^b	+	2	1.3×10^4	4.0×10^5	Slightly pathogenic
32	2.0×10^6	33.3 ± 0.12^b	+	1	1.5×10^4	5.2×10^5	Slightly pathogenic

[1]Highest concentration inoculum of the given strain; [2]+ strain has been reisolated from the heart, – reisolation failed; [3]Number of mice affected from 15; [4] Lethality was calculated at 7 days after inoculation into 15 mice, averages with different superscripts are different at $p<0.05$

Lower impact was observed at strains 3, 18, 23, 26 and 29 with lethality less than 10%. *P. multocida* was successfully recovered from heart blood in most cases, but strains 5, 18, 23 and 29 could not be reisolated. While Collins *et al*. (1983) reported that rabbit isolates growing poorly in liquid medium could be effectively maintained by alternate mouse passage and reisolation by plating on SBA, in our case those strains could not be reisolated. Sick mice did not recover and their conditions worsened gradually; neurological symptoms were observed in these animals. Seven strains were able to cause neurological signs at least on one mouse, 4 affected mice were found at strain 13. These were disturbances in body balance, wobbling and whirling caused by brain lesions. By histopathological evaluation perivascular lymphocytes and lymphocyte infiltration were seen in the cortical parenchyma, and haemorrhagic infiltration in the cortex. Savage and Seldon (1971) reported similar results after intraperitoneal infection of mice with *P. multocida* isolated from rabbit otitis media. The disease developed resembled to torticollis.

The lowest LD_{50} value (1000 CFU/mouse) was found in the case of strain 1, and at the magnitude of 10^5 or 10^6 CFU for strains 5, 9, 13, 18, 22, 31 and 32. The LD_{50} values of the remaining four strains were equal or more than 10^8 CFU/inoculum, which concentration is supposedly higher than it can be in the environment of the rabbits. The inoculums of these strains containing 10^8 or more CFU showed the lowest level of lethality and morbidity. In the experiment of Collins *et al*. (1983) one strain isolated from rabbit with snuffles had an LD_{50} 10^8 CFU for mice infected intraperitoneally and that was a very high value compared to those obtained with those strains which were isolated from turkey and cattle (2 and 100 cells/mouse, respectively).

The outcome of the hierachchical cluster analysis is shown on Figure 2. Cutting the dendrogram at 0.8 similarity level results three homogeneous clusters and two clusters both containing a single strain. Cluster I includes strains 1 and 31, 32. These strains were isolated from dead rabbits and were pathogenic and moderately pathogenic in the mice experiment. On the other hand cluster II and III contain strains different in their mice pathogenecity and by the source rabbit sanitary status. It is apparent furthermore that asymptomatic rabbits could be carriers of *P. multocida* strains moderately pathogenic to mice. These findings confirm the earlier report of Okerman *et al.* (1979) showing that the isolates from rabbits were varying in pathogenecity for mice according to the sanitary status of the source rabbit. Strain 3 isolated from a sick rabbit was not pathogen to mice. The explanation can be that an other aetiological agent causing the disease was implied.

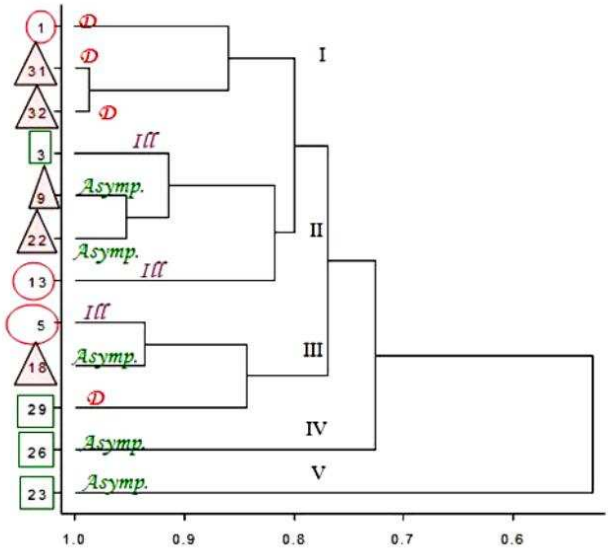

Figure 2: Dendrogram from similarity matrix based on strains pheno- and genotypic caharacters, source rabbit sanitary state and mice pathogenecity indicators. X: similarity level;Y left: mice pathogenecity group, ○ pathogenic, Δ moderately pathogenic, □ non-pathogenic Y right: source rabbit's sanitary status, *D* succumbed, *Ill* suffering in *pasteurellosis*, *Asymp* clinically healthy

CONCLUSIONS

Most of the *P. multocida* strains isolated from sick rabbits or rabbit corpses were also pathogenic in mice. Asymptomatic rabbits probably also can spread slightly pathogenic strains too. After all mice infection with rabbit *P. multocida* strains might not be as informative as it could be expected based on the results of experimental infections with strains isolated from other domestic species.

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